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The SsrA-SmpB Ribosome Rescue System Is Important for Growth of *Bacillus subtilis* at Low and High Temperatures▽

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Bacillus subtilis **has multiple stress response systems whose integrated action promotes growth and survival under unfavorable conditions. Here we address the function and transcriptional organization of a five-gene cluster containing *ssrA*, previously known to be important for growth at high temperature because of the role of its tmRNA product in rescuing stalled ribosomes. Reverse transcription-PCR experiments detected a single message for the *secG-yvaK-rnr-smpB-ssrA* cluster, suggesting that it constitutes an operon. However, rapid amplification of cDNA ends-PCR and *lacZ* fusion experiments indicated that operon transcription is complex, with at least five promoters controlling different segments of the cluster. One σ^A-like promoter preceded *secG* (P_1), and internal σ^A-like promoters were found in both the *rnr-smpB* (P_2) and *smpB-ssrA* intervals (P_3 and P_{HS}). Another internal promoter lay in the *secG-yvaK* intercistronic region, and this activity (P_B) was dependent on the general stress factor σ^B. Null mutations in the four genes downstream from P_B were tested for their effects on growth. Loss of *yvaK* (carboxylesterase E) or *rnr* (RNase R) caused no obvious phenotype. By contrast, *smpB* was required for growth at high temperature (52°C), as anticipated if its product (a small ribosomal binding protein) is essential for tmRNA (*ssrA*) function. Notably, *smpB* and *ssrA* were also required for growth at low temperature (16°C), a phenotype not previously associated with tmRNA activity. These results extend the known high-temperature role of *ssrA* and indicate that the ribosome rescue system is important at both extremes of the *B. subtilis* temperature range.**

Bacteria must coordinate multiple stress response networks to grow and survive under changing environmental conditions. Here we focus on the transcriptional organization and stress-related function of the *secG-yvaK-rnr-smpB-ssrA* gene cluster in *Bacillus subtilis*. We were drawn to this cluster by transcriptional profiling experiments designed to identify elements of the general stress regulon controlled by σ^B, which is activated by diverse stimuli to provide broad resistance against future challenges (reviewed in references 20 and 46). The profiling studies indicate that the adjacent *yvaK* and *rnr* genes are under σ^B control (8, 22, 45, 47), and from sequence inspection, two of the studies suggested that a σ^B-dependent promoter precedes *yvaK* (22, 45). However, this promoter was sufficiently divergent from most well-defined σ^B promoters to be missed by a hidden Markov model analysis (47), and it has not been experimentally characterized.

As we shall describe elsewhere, we pooled and reanalyzed the available profiling data in an effort to identify new elements of the σ^B regulon that may have escaped detection in the individual studies (P. Fawcett, A. Weigel, and C. W. Price, unpublished data). Of relevance here, the reanalysis suggested that, in addition to *yvaK* and *rnr*, the adjacent *smpB* gene was also partly under σ^B control. Given that the only rho-independent terminator sequence for the five-gene cluster lies downstream from *ssrA*, the reanalysis left open the possibility that *ssrA* expression was also influenced by σ^B. This regulation would have been overlooked by the profiling studies, which did not include *ssrA* in their arrays because it does not encode a protein product.

The *ssrA* gene codes for tmRNA, which functions as both a tRNA and an mRNA to rescue ribosomes that are unproductively stalled on a message (reviewed in references 31 and 59). As part of this *trans*-translation process, a peptide tag is added to the C-terminal end of the released protein, thus targeting it for proteolysis (17, 35, 58). The presence of *ssrA* in all of the sequenced prokaryotic genomes indicates the biological importance of the *trans*-translation system (31). However, *ssrA* is known to be essential in only a few species, and for enteric bacteria and *B. subtilis* its loss is largely manifested in stress-related phenotypes. For example, an *Escherichia coli ssrA* mutant has a decreased ability to grow at 45°C (36) and also exhibits some regulatory anomalies and a variety of phage developmental phenotypes (31, 59). In another example, loss of *ssrA* function significantly affects pathogenesis in *Salmonella enterica* and *Yersinia pseudotuberculosis*, in part because of an inability of the mutants to survive in macrophages (4, 29, 43). And in *B. subtilis*, *ssrA* is important for growth at 52°C or under conditions of cadmium or ethanol stress (41). In all of the cases tested, *ssrA* activity requires the SmpB protein, which mediates the binding of *ssrA* to the ribosome and performs at least one additional downstream function in the *trans*-translation process (33, 34, 52, 58).

Given that the *ssrA* gene is known to have stress-related functions in *B. subtilis*, control of its expression by the general stress factor σ^B was an intriguing possibility. However, the work of Muto et al. (41) indicated the presence of a heat shock promoter in the *smpB-ssrA* intercistronic region, and additional promoters controlling the cluster could not be excluded. We therefore focused on the transcriptional organization of

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TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype	Reference or construction[a]
PB2	*trpC2*	168 Marburg strain
PB105	*sigBΔ1 trpC2*	30
PB153	*sigBΔ2*::*cat trpC2*	6
PB344	*sigBΔ3*::*spc trpC2*	5
PB918	*amyE*::pJH2 *trpC2*	pJH2 → PB2
PB919	*amyE*::pJH2 *trpC2*	pJH2 → PB344
PB970	*amyE*::pJH3 *trpC2*	pJH3 → PB2
PB972	*amyE*::pJH5 *trpC2*	pJH5 → PB2
PB973	*amyE*::pJH10 *trpC2*	pJH10 → PB2
PB978	*amyE*::pJH1 *trpC2*	pJH1 → PB2
PB979	*amyE*::pJH7 *trpC2*	pJH7 → PB2
PB981	*amyE*::pJH1 *trpC2*	pJH1 → PB344
PB982	*amyE*::pJH15 *trpC2*	pJH15 → PB2
PB990	*yvaK*::pJH11 *trpC2*	pJH11 → PB2
PB992	*rnr*::pJH12 *trpC2*	pJH12 → PB2
PB1002	*ssrA*::*cat trpC2*	D1 → PB2
PB1017	*trpC*$^+$	pTRP-H3 (3) → PB2
PB1018	*sigBΔ1 trpC*$^+$	pTRP-H3 → PB105
PB1019	*smpBΔ1 trpC*$^+$	pTRP-H3 + pJH13 → PB2[b]
PB1020	*ssrA*::*cat trpC*$^+$	pTRP-H3 → PB1002
PB1021	*ssrA*::*cat amyE*::*ssrA trpC*$^+$	pJH14 → PB1020
D1	*ssrA*::*cat pur met his*	41

[a] Arrow indicates transformation from donor to recipient.
[b] Congression of *smpBΔ1* borne on linearized pJH13 with *trpC*$^+$ on pTRP-H3.

the *secG-ssrA* region with the aim of locating its principal promoter activities. This analysis found at least five, including a weak σ^B-dependent promoter preceding *yvaK* and also strong σ^A-like promoters preceding *smpB* and *ssrA* that would facilitate their differential expression. These data, in turn, allowed us to make nonpolar null mutations to probe the importance of individual gene products for growth at the extremes of the *B. subtilis* temperature range. On the basis of these results, we suggest that the genes in the *secG-ssrA* cluster form a functional module beneficial to members of the order *Bacillales*.

MATERIALS AND METHODS

Bacterial strains and genetic methods. The *B. subtilis* strains used in this study are listed in Table 1. Standard recombinant DNA methods were according to reference 49, and transformation of *B. subtilis* PB2 and derivatives was according to reference 14. Plasmids used for these constructions are shown in Table 2. To locate promoter activity, fragments were PCR amplified and cloned into transcriptional fusion vector pDG268, which integrates in single copy at the *amyE* locus (2). To make in-frame *smpBΔ1*, removing triplets 4 to 153, a four-primer fragment (24) was cloned into the pCP115 vector (6) to create pJH13; *smpBΔ1* was then substituted for the wild-type allele via a two-step replacement procedure (51). To complement the *ssrA*::*cat* insertion in *trans*, a fragment was PCR amplified and cloned into the pDG1730 vector (19), placing P_3, P_{HS}, and *ssrA* in single copy at *amyE*. DNA sequences were confirmed for all of the constructs.

TABLE 2. Plasmid constructions used in this study

Plasmid	Primer[a]	Vector (reference) and sites
pJH1	F, 5′ CATATGAATAGGGTAACCAAG 3′ R, 5′ ACCGCTTTGTCTCCGCCT 3′	pDG268[b] (2); EcoRI-BamHI
pJH2	F, 5′ TCAGGCCTTTTTCACGTT 3′ R, 5′ ACCGCTTTGTCTCCGCCT 3′	pDG268[b]; EcoRI-BamHI
pJH3	F, 5′ AGCGAATTCTGAGGAAAAAGGGGA 3′ R, 5′ GGTGGATCCTATAAAATAATCGTGG 3′	pDG268; EcoRI-BamHI
pJH5	F, 5′ GAGGAATTCGCAAAAGGTGGACAG 3′ R, 5′ GGTGGATCCTATAAAATAATCGTGG 3′	pDG268; EcoRI-BamHI
pJH7	F, 5′ GCTGAATTCGCACCGCAAGGAGAT 3′ R, 5′ CTGGGATCCTCTTCTTACGTTCTCA 3′	pDG268; EcoRI-BamHI
pJH10	F, 5′ CCGGAATTCCCTTATACCAAGGGG 3′ R, 5′ CTGGGATCCTCTTCTTACGTTCTCA 3′	pDG268; EcoRI-BamHI
pJH11 (*yvaK*)	F, 5′ CCCAAGCTTATGGCGTCCCGCCTGAAG 3′ R, 5′ CGCGGATCCTGAGGGTATTCATCGGCG 3′	pMUTIN4 (53); HindIII-BamHI
pJH12 (*rnr*)	F, 5′ CCCAAGCTTGCAATGAACGGCGATATC 3′ R, 5′ CGCGGATCCGAATGGTTTCAACCTCAC 3′	pMUTIN4; HindIII-BamHI
pJH13 (*smpBΔ1*)	P1 5′ GGAGAATTCCATGTCAGCTTTATGAC 3′ P2 5′ ACTATGCCTTTAGAAGCCTTTTGGCATGCCAGAACC 3′ P3 5′ GGCTTCTAAAGGCATAGTGCT 3′ P4 5′ GGTGGATCCCGAACCCACGTCCAGAAA 3′	pCP115 (6); EcoRI-BamHI
pJH14 (P3-*ssrA*)	F, 5′ GGGGGATCCAACTATGACAAACGGGAAG 3′ R, 5′ GGTGAATTCTCTGCTCTGCAATCAGTATG 3′	pDG1730 (19); BamHI-EcoRI
pJH15	F, 5′ CACGAATTCATCACGACGCCATTC 3′ R, 5′ CTGGGATCCTCTTCTTACGTTCTCA 3′	pDG268; EcoRI-BamHI

[a] F, forward; R, reverse. Sequences corresponding to restriction sites are underlined.
[b] Fragments were blunt end ligated into HincII-cut pUC19 and then excised with EcoRI and BamHI.

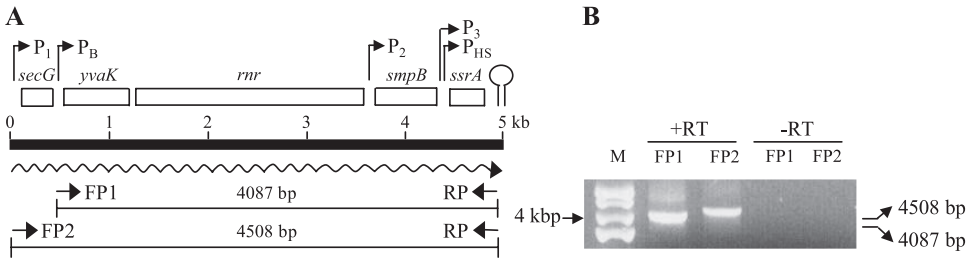


FIG. 1. The five genes in the *secG-ssrA* cluster can be transcribed as a polycistronic message. (A) Map of the *B. subtilis secG-ssrA* region showing promoter activities located in this work (P_1, P_B, P_2, and P_3), the heat shock promoter described by Muto et al. (P_{HS}; reference 41), and the proposed rho-independent terminator sequence following *ssrA*. A full-length transcript of the region is drawn beneath the kilobase scale, together with the predicted sizes of the RT-PCR products resulting from its amplification with primers FP1, FP2, and RP. (B) RT-PCR full-length transcript detection in total RNA isolated from wild-type *B. subtilis*. Lanes +RT and −RT indicate with and without reverse transcriptase, lane M contains size markers, and the predicted lengths of the two RT-PCR products are on the right.

Reverse transcription (RT)-PCR and rapid amplification of cDNA ends (RACE)-PCR experiments. Shake cultures were grown at 37°C to mid-exponential phase in buffered Luria broth (BLB) medium lacking salt (5) and then diluted 1:25 into fresh BLB medium. At mid-exponential phase, some cultures were subjected to a 10-min ethanol stress (5%, vol/vol) while others were allowed to grow untreated before harvesting and RNA extraction. Total RNA was isolated with the RNeasy kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions, except that the lysozyme concentration was increased to 15 mg/ml and a homogenization step (passage through a 20-gauge needle) was added.

For RT-PCR, RNA was extracted from wild-type (PB2) cells and the RT reaction was done according to reference 49. One microgram of total RNA and 25 pmol of gene-specific primer 5′-GATTACTTAAGCGTCTACG-3′ were incubated at 65°C for 10 min, and then Transcriptor reverse transcriptase (Roche Applied Science, Mannheim, Germany) was added and incubation was continued at 50°C for 90 min. The resulting cDNA was amplified in a PCR with *Taq* polymerase (Promega, Madison, WI). As a negative control, the RT reaction was performed without the addition of reverse transcriptase. To detect a *yvaK*-to-*ssrA* complement, the forward primer FP1 (5′-CGGATGTAAGGATGCTGGGA-3′) was used with the reverse primer RP (5′-GGTTTCACTCATCTTCTTGCC-3′); to detect a *secG*-to-*ssrA* complement, the FP2 forward primer (5′-GGTTATCG TCAGCATTGC-3′) was used with the same reverse primer. PCR products were analyzed by agarose gel electrophoresis.

For RACE-PCR (16), RNA was extracted from wild-type (PB2) or *sigB* mutant (PB153) cells. RACE-PCRs were done as previously described (47), with a primer complementary to the poly(A) tail (5′-GACCACGCGTATCGATGTC $GACT_{16}$V-3′ [where V = A, C, or G]) and two nested, gene-specific primers, (i) YVAK1 (outer, 5′-GTATGTACAAGTTCTTCAGGC-3′) and YVAK2 (inner, 5′-GACGCCATGTCCTTCATATTGAG-3′); (ii) SMPB1 (5′-GTTATAGCGG TTTCCCTG-3′) and SMPB2 (5′-GGGCTGACGTGCATATTGTGG-3′); or (iii) SSRA1 (5′-TCTTCTTACGTTCTCAGA-3′) and SSRA2 (5′-CGCAAGCG TAGCCTACTTGGA-3′). PCR products were separated on agarose gels, purified and sequenced.

β-Galactosidase accumulation assays. Shake cultures were grown at 37°C in BLB medium and diluted 1:25 into fresh medium as described for the RNA experiments above. At mid-exponential phase, cultures were subjected to ethanol stress (5%, vol/vol). Samples were collected at the indicated times and assayed according to Miller (39), by using sodium dodecyl sulfate and chloroform to permeabilize the cells. Protein levels were determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Activity was defined as $\Delta A_{420} \times 1{,}000$ per minute per milligram of protein.

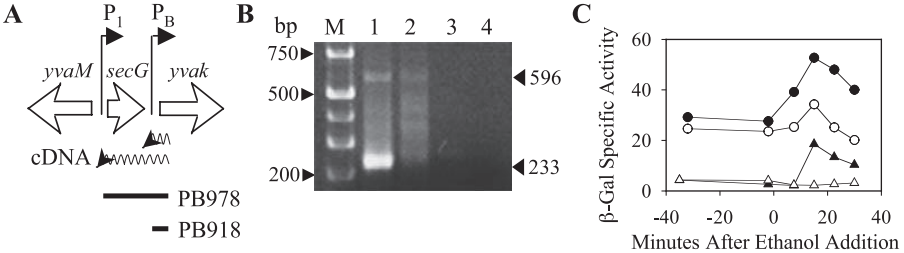
Growth experiments. High-temperature growth experiments were done essentially as described by Holtmann et al. (26), and low-temperature experiments were done essentially as described by Brigulla et al. (7). For high-temperature experiments, wild-type and mutant cells were precultured at 37°C in shake flasks containing either BLB medium or Spizizen's minimal medium with 0.5% glucose (SMM; reference 1). At $A_{578} = 0.5$, exponentially growing cells were diluted 1:5 into fresh BLB medium or SMM and transferred to a 52°C water bath shaker. For low-temperature experiments, cells were similarly precultured in SMM, diluted 1:5 into fresh SMM, and transferred to a 16°C air shaker. Growth was monitored with a Klett-Summerson colorimeter with a number 66 (red) filter; units were plotted on a linear scale versus time, in accordance with references 7 and 26.

RESULTS

The *secG-ssrA* gene cluster can be transcribed as a single polycistronic message. As shown in Fig. 1A, the *secG-ssrA* region encodes only a single rho-independent terminator sequence that lies downstream from *ssrA*, leading us to hypothesize that the five-gene cluster could be transcribed as a single message. We used RT-PCR to look for such a message in RNA isolated from unstressed, exponential-phase *B. subtilis* cells. Two different forward primers were designed, one to anneal to the *secG* message and the other to anneal to the *yvaK* message. In conjunction with a reverse primer designed to anneal to the *ssrA* message, the *secG* primer was expected to amplify a product of 4,508 bp and the *yvaK* primer was expected to amplify a product of 4,087 bp.

As shown in Fig. 1B, products of the predicted size were found in the reaction mixtures with reverse transcriptase. These products were missing from the reaction mixtures lacking transcriptase, ruling out the possibility that they arose from amplification of contaminating DNA. We conclude that at least some full-length message is transcribed from the *secG-ssrA* cluster and that these genes constitute an operon. However, further analysis revealed the existence of multiple promoters within this region, some stress inducible. These promoters fell into three sets: (i) two in the *secG-yvaK* region, (ii) one in the *rnr-smpB* interval, and (iii) two in the *smpB-ssrA* interval.

The *secG-yvaK* region. Expression profiling experiments previously identified *yvaK* as a candidate element of the σ^B regulon (22, 45, 47); its product is a carboxylesterase of unknown function (23). While a putative σ^B-dependent promoter for *yvaK* could be located by inspection, it has not been characterized experimentally. With RNA isolated from cells that had been ethanol stressed to increase σ^B activity, RACE-PCR experiments found several potential 5′ ends for the *yvaK* message. We could obtain sequences from the two most prominent PCR products (Fig. 2A and B), locating one 5′ end to a site 24 nucleotides (nt) upstream from the ATG initiation triplet of *yvaK* (Fig. 2E) and the other to a site 31 nt upstream from the ATG of *secG* (Fig. 2D). The *yvaK* 5′ end was preceded by sequences previously suggested to define the σ^B-dependent promoter, with GtTTTt at −35 and tGGaAa at −10 (22, 45); this 5′ end was not apparent when RNA extracted from etha-



D

catcgcttttctcctttgacctttttcatatgaatagggtaaccaagataaaacgtcttatccggccttttggcgtctgat
-35 -10 ▼(P_1) *secG* start
acagc**gtgaca**tgcaaccctttttcatg**taaaat**agaagt**a**atgtagccagtgagtctggaggtgtatggg**atg**cacgcag

E

secG stop -35
tagggcaatgtttgtataaggtctgatgtgaagtcaggccttttttcacgtttctggatgatattcaaaac**gttttt**ttct
-10 ▼(P_B) *yvaK* start
gattaaactg**tggaaa**actaaaat**g**atcgtgcagatagaaagggagac**atg**agcatgaaagttgtgacaccaaaacaatt

FIG. 2. One σ^A-like and one σ^B-dependent promoter in the *secG-yvaK* region. (A) Diagram showing promoter location, cDNA from the RACE-PCR experiments, and fragments fused to a *lacZ* reporter for in vivo assay. (B) RACE-PCR products of RNA extracted from ethanol-stressed cells of wild-type PB2 (lane 1) or *sigB* null mutant PB153 (lane 2), with negative controls in which the poly(dA) tailing step was omitted for first-strand synthesis products from wild-type (lane 3) or *sigB* mutant (lane 4) RNA. Size markers are on the left, and the positions of the 596- and 233-bp products (determined by direct DNA sequencing) are indicated on the right. The 5′ ends of these products are located on the DNA sequences in panels D and E. (C) Promoter activity arising from the fragments shown in panel A was measured indirectly by β-galactosidase (β-Gal) accumulation from *lacZ* transcriptional fusions. Early-exponential-phase cells were stressed by the addition of 5% ethanol at time zero. Longer fragment: filled circles, PB978 (wild type); open circles, PB981 (*sigB*Δ*3*). Shorter fragment: filled triangles, PB918 (wild type); open triangles, PB919 (*sigB*Δ*3*). (D) *yvaM-secG* intercistronic region, with the translation start sites in bold (cat for divergently transcribed *yvaM* and atg for *secG*). The 5′ end of the 596-bp product from panel B is marked by a filled inverted triangle (P_1), with potential −35 and −10 sequences for σ^A in bold and underlined. (E) *secG-yvaK* intercistronic region, with the *secG* termination site (tag) and the *yvaK* start site (atg) in bold. The 5′ end of the 233-bp product from panel B is marked by a filled inverted triangle (P_B), with potential −35 and −10 sequences for σ^B in bold and underlined.

nol-stressed cells of a *sigB* null mutant was used (Fig. 2B). We provisionally call this promoter P_B. By contrast, the *secG* 5′ end was preceded by sequences resembling a σ^A promoter, with gTGACA at −35 and TAaAAT at −10. This signal was diminished but still detectable in the *sigB* null mutant, while more rapidly migrating minor bands increased in intensity, suggesting that they were decay products of the *secG* message. We provisionally call this promoter P_1. The 5′ end associated with P1 was found with a primer within *yvaK*, indicating that the *secG* message extends into the *yvaK* reading frame.

To test if the *secG-yvaK* region contained functional promoter activities, we made two transcriptional fusions to a *lacZ* reporter gene (Fig. 2A). One carried a fragment extending from the divergently transcribed *yvaM* gene preceding *secG* to a site within *yvaK*; this would be expected to contain both the P_1 and P_B activities suggested by RACE-PCR. The other carried a shorter fragment that removed 387 bp from the 5′ end of the first; this would be expected to contain only P_B activity. These fusions were placed in single copy at the *amyE* chromosomal locus. As shown in Fig. 2C, in the wild type the longer fusion manifested promoter activity in unstressed cells, and this activity increased following ethanol stress. In the *sigB* null mutant, this same fusion had slightly less activity in unstressed cells and only a modest increase following stress. By contrast, in the wild type the shorter fusion had very low activity in unstressed cells and a significant increase following ethanol stress, as is the case with well-characterized σ^B-dependent fusions. Moreover, this stress-induced increase in activity was abolished in the *sigB* null mutant. The fusion data are consistent with the RACE-PCR assays and led us to conclude that at least two promoters lie in the *secG-yvaK* region, i.e., a σ^B-independent P_1 activity preceding *secG* and a σ^B-dependent P_B activity preceding *yvaK*. On the basis of the β-galactosidase accumulation assays, P_B appears to be relatively weak, consistent with its sequence divergence from more typical σ^B-dependent promoters.

The *rnr-smpB* interval. A similar analysis located promoter activity in the *rnr-smpB* interval. RACE-PCR produced a single abundant product of about 290 bp that did not appear to differ quantitatively among the three sources of RNA—wild-type cells with or without ethanol stress and *sigB* mutant cells with ethanol stress (Fig. 3A and B). Sequencing of the PCR product located its 5′ end to a site 47 nt upstream from the ATG initiation triplet of *smpB* (Fig. 3D). This 5′ end was preceded by sequences resembling an extended σ^A promoter (21), here provisionally called P_2, with TTGtag at −35 and TGgTAaAAT at −10. We made two *lacZ* fusions to determine whether the region containing these sequences had promoter activity (Fig. 3A). The first carried a fragment extending from within the 748th triplet of the upstream *rnr* gene to a site within *smpB*, and the second removed 198 bp from the 5′ end of this fragment, deleting the proposed −35, −10, and +1 sequences of P_2. The longer fragment indeed contained promoter activity, and this increased less than twofold in ethanol-stressed cells (Fig. 3C). By contrast, the fusion bearing the shorter fragment had no detectable activity. From the RACE-PCR and fusion results, we conclude that the *rnr-smpB* interval contains at least one promoter, P_2, which is modestly induced by ethanol stress.

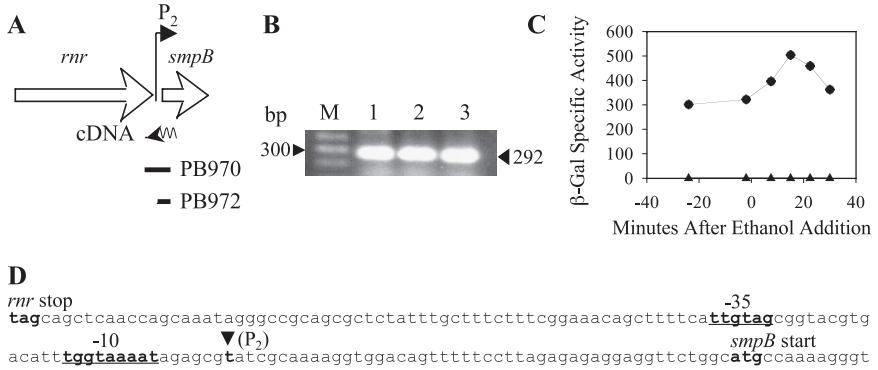


FIG. 3. One σ^A-like promoter in the *rnr-smpB* interval. (A) Diagram of interval labeled as in the Fig. 2 legend. (B) RACE-PCR products of RNAs extracted from ethanol-stressed cells of wild-type PB2 (lane 1) or *sigB* null mutant PB153 (lane 2) and from unstressed wild-type cells (lane 3). Size markers are on the left, and the 292-bp product is labeled on the right; the 5′ end of this product is located on the sequence in panel D. (C) Promoter activity from the fragments shown in panel A measured by β-galactosidase (β-Gal) accumulation as described in the Fig. 2 legend. Longer fragment: filled circles, PB970. Shorter fragment: filled triangles, PB972. (D) *rnr-smpB* intercistronic region, with the *rnr* termination site (tag) and the *smpB* start site (atg) in bold. The 5′ end of the 292-bp product from panel B (determined by DNA sequencing) is marked by a filled inverted triangle (P_2), with potential −35 and −10 sequences for an extended σ^A promoter in bold and underlined.

The *smpB-ssrA* interval. Because of the rapid *ssrA* transcript processing to yield mature 10S tmRNA, fusion analysis was key in locating two potential promoter activities in the *smpB-ssrA* interval. RACE-PCR produced three products of 241, 295, and 370 bp from RNAs isolated from ethanol-stressed wild-type and *sigB* mutant cells (Fig. 4A and B, lanes 1 and 2). Of these, the 370-bp product appeared to be reduced or absent when RNA from unstressed wild-type cells was used (Fig. 4B, lane 3). DNA sequencing was able to locate the 5′ end for the 241-bp principal product, which was the 5′ end of the mature 10S tmRNA (Fig. 4D). However, no sequence data could be obtained for the 295- and 370-bp minor products, so we estimated the locations of their 5′ ends on the basis of gel mobility. The 295-bp product appeared to correspond to a transcript from the *ssrA* heat shock promoter mentioned by Muto and colleagues (41); its estimated 5′ end lay downstream from sequences resembling an extended σ^A promoter, here called P_{HS}, with TTGAaA at −35 and TGtTATAAT at −10 (Fig. 4D). The 370-bp product had an estimated 5′ end that lay downstream from another set of sequences resembling an extended σ^A promoter, here provisionally called P_3, with TTGAtt at −35 and TGcTATAcT at −10 (Fig. 4D).

We made three *lacZ* fusions to determine whether the sequences containing P_3 and P_{HS} were important for promoter activity (Fig. 4A). The first carried a fragment extending from within the 86th triplet of the upstream *smpB* gene to a site within *ssrA*; the second removed 269 bp from the 5′ end of this fragment, deleting the proposed −35, −10, and +1 sequences for P_3; and the third removed an additional 110 bp, deleting the proposed −35, −10, and +1 sequences for P_{HS}. The long-

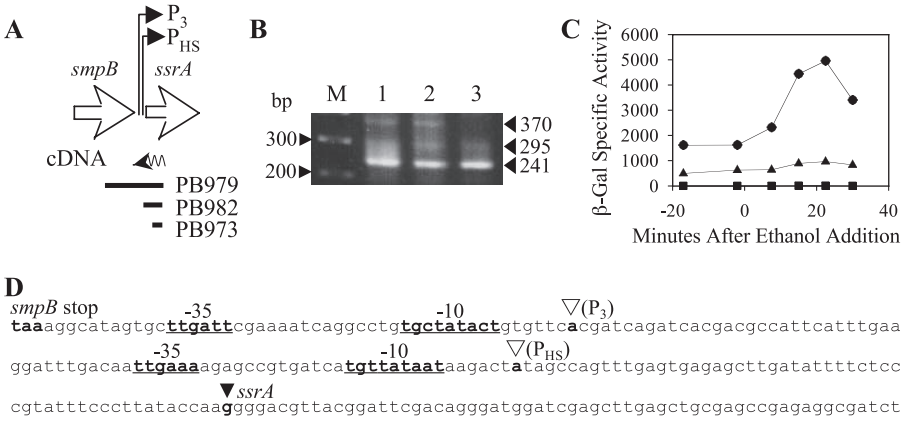


FIG. 4. Two σ^A-like promoter activities in the *smpB-ssrA* interval. (A) Diagram labeled as in the Fig. 2 legend. (B) RACE-PCR products of RNAs extracted from ethanol-stressed cells of wild-type PB2 (lane 1) or *sigB* null mutant PB153 (lane 2) and from unstressed wild-type cells (lane 3). Size markers are on the left, and the 241-, 295-, and 370-bp products are labeled on the right. (C) Promoter activity from the fragments shown in panel A, measured as described in the Fig. 2 legend. Longest fragment: filled circles, PB979. Middle fragment: filled triangles, PB982. Shortest fragment: filled squares, PB973. β-Gal, β-galactosidase. (D) *smpB-ssrA* intercistronic region, with the *smpB* termination site (taa) and the 5′ end (g) of the mature tmRNA in bold. The estimated 5′ ends of the 370- and 295-bp products from panel B are marked by open inverted triangles (P_3 and P_{HS}, respectively), with potential −35 and −10 sequences for extended σ^A promoters in bold and underlined. The 5′ end of the 241-bp product from panel B (determined by DNA sequencing) is indicated by the filled inverted triangle (*ssrA*) and represents the processed (or mature) 10S tmRNA.

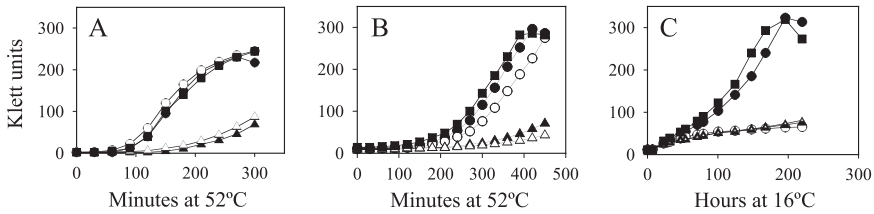


FIG. 5. Growth of an *smpB* or *ssrA* mutant is impaired at both high and low temperatures. For high-temperature experiments, cells were grown at 37°C in shake flasks containing either BLB medium or SMM. At A_{578} = 0.5, cells were diluted 1:5 into fresh BLB medium (A) or SMM (B) and transferred to a 52°C water bath shaker. For low-temperature experiments, cells were grown in SMM, diluted 1:5 into fresh SMM (C), and transferred to a 16°C air shaker. All strains were Trp^+ prototrophs to avoid possible transport effects. Filled circles, PB1017 (wild type); open circles, PB1018 (*sigBΔ1*), which served as a negative control in the low-temperature experiments (28); filled triangles, PB1019 (*smpBΔ1*); open triangles, PB1020 (*ssrA*::*cat*); filled squares, PB1021 (*ssrA*::*cat* with P_3, P_{HS}, and *ssrA* in *trans*).

est fragment had strong promoter activity, and this increased about threefold in ethanol-stressed cells (Fig. 4C). With the second fragment, this activity significantly decreased, consistent with the loss of P_3, and ethanol induction was no longer apparent. With the third fragment, there was no detectable activity, consistent with the loss of P_{HS}. From the fusion results shown in Fig. 4C, P_3 is ethanol inducible, a conclusion in accord with the signals present in the RACE-PCR experiment shown in Fig. 4B (compare the strengths of the 370-bp signals in lanes 1 and 3). From the sum of the RACE-PCR and fusion results, we conclude that the *smpB-ssrA* interval contains at least two promoter activities, P_3 and P_{HS}.

Loss of *smpB* or *ssrA* function has a significant effect on growth at both high and low temperatures. Muto et al. (41) previously showed that *ssrA* function was required for robust growth of *B. subtilis* at high temperature and under conditions of ethanol or cadmium stress. Moreover, additional genes in the *B. subtilis* cluster are known to have stress- or tmRNA-related functions in other bacteria. For example, RNase R is a processive 3′-to-5′ exonuclease that can degrade RNA with significant secondary structure (11, 44); it has been implicated in quality control of rRNA, as well as degradation of certain aberrant mRNAs recognized by the *trans*-translation system (12, 48). This latter activity is consistent with the copurification from *E. coli* extracts of a complex containing tmRNA, SmpB, and substoichiometric amounts of RNase R (32). A role for *E. coli* RNase R in adapting to environmental transitions is suggested by its elevated level following cold stress, starvation, or entry into stationary phase (9, 10).

In order to uncover their possible roles in stress resistance, we constructed null alleles in the four genes downstream from the P_B promoter in the *B. subtilis* cluster and tested their effects on growth at high and low temperatures. It proved difficult to make strains bearing in-frame deletions within *yvaK* or *rnr*, so these were built with the pMUTIN4 plasmid, which disrupts the gene of interest while ensuring continued transcription of downstream genes (53). We were able to construct a strain bearing a deletion (*smpBΔ1*) that removed most of the *smpB* coding region; this in-frame deletion did not affect the initiation or termination signals for *smpB* transcription and translation. We also moved the *ssrA*::*cat* insertion of Muto et al. (41) into our genetic background so that all of the constructed strains were isogenic (Table 1).

In agreement with the results of Muto et al. (41), we found that the *ssrA*::*cat* insertion had a major impact on growth when cells were shifted from 37 to 52°C and that this phenotype was complemented in *trans* by a fragment containing P_3, P_{HS}, and *ssrA* (Fig. 5A and B). We also established that the *smpBΔ1* null allele had the same high-temperature phenotype, as expected if SmpB is required for *ssrA* function (33, 34, 58). Our significant new findings are that (i) *ssrA*::*cat* had an equally striking impact on growth when cells were shifted from 37 to 16°C; (ii) this low-temperature phenotype could be fully complemented by a fragment containing P_3, P_{HS}, and *ssrA*; and (iii) the *smpBΔ1* allele had the same phenotype (Fig. 5C). *smpBΔ1* is an in-frame deletion that should not affect the expression of downstream genes, and the complementation test indicated that loss of *ssrA* function alone was sufficient to cause the low-temperature phenotype. Together, these results argue that it was the absence of a functional ribosome rescue system and not the production of a tmRNA fragment from the interrupted *ssrA*::*cat* allele that had the harmful effect. We conclude that *smpB* and *ssrA* are each required for robust growth at both low and high temperatures and that the promoters on the P_3-P_{HS}-*ssrA* fragment provide sufficient *ssrA* expression to support growth under both conditions.

In contrast, the *yvaK* or *rnr* null allele had no substantial effect on growth at either temperature extreme (data not shown). These *rnr* results are in agreement with the earlier study of Höper et al. (28), who noted that loss of *rnr* function had no significant effect on growth or survival in heat or cold.

DISCUSSION

It is uncommon for loss of a single cellular system to have only a slight impact under optimal conditions while significantly affecting growth at both temperature extremes, as we have shown here for the *B. subtilis trans*-translation apparatus encoded by *smpB* and *ssrA*. To our knowledge, the only comparable example is the ability of *B. subtilis* to transport compatible solutes, which help maintain protein functionality at both the 52°C maximum growth temperature and the 15°C standard used for cold stress experiments (7, 25). The striking phenotype caused by loss of *smpB* or *ssrA* function reflects the importance of maintaining cellular translational capacity at the extremes of the temperature range.

At optimal temperatures, absence of a *trans*-translation system reduces the growth rates of *E. coli* and *B. subtilis* only marginally, if at all (33, 36, 41). This absence of a strong phenotype is likely due to a low level of stalling under these

conditions and also to the presence of alternative routes of spontaneous or factor-dependent recycling that result in peptidyl-tRNA drop off, a process which ultimately requires peptidyl-tRNA hydrolase to restore the affected tRNA (17, 38, 40, 58). However, *trans*-translation is more efficient in releasing stalled ribosomes and in reducing the peptidyl-tRNA load (50). Its loss would therefore become more telling at maximum growth temperatures, when unbalanced tRNA pools and a sharp increase in truncated messages significantly increase stalling. Absence of *trans*-translation in *B. subtilis* has a greater impact on high-temperature growth than is the case for *E. coli* (33, 36, 41). This difference may reflect a decreased ability of the alternative routes of ribosome recycling or of peptidyl-tRNA hydrolysis to cope with high-temperature demand in *B. subtilis*.

We have now shown that absence of *trans*-translation in *B. subtilis* has a significant impact on low-temperature growth (Fig. 5), another phenotype not strongly manifested in *E. coli* (33). What is the role of this system at low temperature? Inefficient translational initiation appears to be the rate-limiting step for bacterial growth in the cold (15), and production of helicases or cold shock proteins is a known adaptation by which to cope with the increased RNA secondary structure that contributes to this low rate (57). On the basis of the results presented here, we suggest that recycling of stalled ribosomes by *trans*-translation also makes a critical contribution to initiation in cold-stressed *B. subtilis*. This quality control system would both maintain the pool of actively translating ribosomes and release needed amino acids, thereby facilitating synthesis of the proteins required to adapt to cold conditions.

What is the significance of the gene arrangement found in the *secG-ssrA* region? This particular organization does not appear to be explained by a need for coregulation. Although all five genes can be transcribed as a single message, presumably from the P_1 promoter preceding *secG*, internal promoters allow differential expression of individual genes and sets of genes. Assuming that our β-galactosidase assays roughly correlate with promoter strength, P_2 (preceding *smpB*) and P_3/P_{HS} (preceding *ssrA*) are particularly strong. We speculate that the strength of these promoters and the translation efficiency of the *smpB* message have coevolved such that SmpB and tmRNA are produced in a physiologically appropriate stoichiometry during unstressed growth at mesophilic temperatures. If this is the case, the increased transcription of *ssrA* from P_3 and P_{HS} following ethanol or heat stress (Fig. 4; reference 41) implies that tmRNA has an SmpB-independent function or that tmRNA is less stable under these conditions. The latter possibility was first advanced in the model of Hong et al. (27), who suggested that *E. coli* tmRNA is degraded following its unfolding or cleavage under stress conditions. For example, diverse stresses are known to activate the *E. coli* RelE and MazF toxins to induce cleavage of both mRNA and tmRNA (see reference 18 and references therein). Toxin-induced cleavage of mRNA is thought to assist stress adaptation by recycling and redirecting ribosomes as needed, but the accompanying cleavage of tmRNA seems to be counterproductive for this adaptation process. Thus, if transcription of *B. subtilis ssrA* is indeed increased relative to *smpB*, it may serve to compensate for such a stress-induced instability. A detailed investigation of SmpB and tmRNA synthesis and stability addressed the important role of these processes in controlling cell cycle progression in *Caulobacter crescentus* (27). On the basis of the initial results reported here and by others (41), a similar study of stress-induced changes in the *B. subtilis* system may be warranted.

There seems to be little physiological need for transcription from the relatively weak P_B promoter preceding *yvaK* to read into *ssrA*, for the simple reason that the fragment containing P_3/P_{HS} had sufficient promoter activity to fully complement in both heat and cold (Fig. 5). Although we cannot rule out the possibility that P_B-directed transcription into *ssrA* plays a role under special conditions, it seems that P_B primarily serves to increase *yvaK*, *rnr*, and possibly *smpB* expression under the same circumstances that promote induction of *E. coli rnr*, i.e., starvation, entry into stationary phase, or cold stress (9, 10). Indeed, an *E. coli rnr* mutant has a cold stress phenotype (9). However, the absence of a similar phenotype for a *B. subtilis rnr* mutant suggests that this activity is not essential under cold conditions or that its stress-related function partly overlaps that of another RNase.

Although a need for coregulation does not appear to have strongly shaped the genetic organization of the *secG-ssrA* region, the clustering itself supports an inference of a functional relationship among seemingly diverse gene products (56). For example, products that associate in a complex are more likely to be encoded by clustered genes (13), and this driving force may be part of the basis for the *rnr-smpB-ssrA* arrangement (32, 48). However, physical association does not seem to explain the presence of *secG* or *yvaK* in the cluster. *secG* encodes an auxiliary membrane protein that interacts with the SecA-SecE-SecY translocase; its loss causes a growth defect under conditions in which membrane function is compromised, such as at low temperature or under high secretory demand (54). And *yvaK* encodes carboxylesterase E, which hydrolyzes short- and medium-length fatty acid esters in vitro (23); its in vivo substrate and function are unknown. Nonetheless, analysis of sequenced genomes indicates that a conserved gene order is one of the strongest predictors of a functional module in which dissimilar gene products together affect a common cellular process (56). Horizontal gene transfer could provide the selection for the assembly and maintenance of such a cluster, thereby facilitating the cotransfer of complex capabilities (reviewed in reference 37). On the basis of the cold-sensitive phenotypes of *secG* and *smpB*/*ssrA* mutants, we suggest that the cluster represents a functional module that confers enhanced adaptation to cold stress. In this view, the YvaK carboxylesterase would also contribute to adaptation, a suggestion reinforced by the common regulation of *yvaK* and *rnr* by the general stress factor σ^B. Such an enhanced adaptation might be especially important if the capacity of the *B. subtilis trans*-translation system is less than that of *E. coli*, as has been suggested (40).

Evidence for conservation of the *secG-ssrA* cluster and suggestion of its horizontal transfer can be found in the STRING database, version 6.3 (55), which shows this particular cluster to be largely restricted to the order *Bacillales*. Here the five-gene cluster is exactly conserved among some representatives, such as *Bacillus cereus* and *Staphylococcus aureus*, but not in others, such as *Listeria monocytogenes*, in which *ssrA* lies at a different locus. From the absolute conservation of the *secG-*

yvaK-rnr-smpB arrangement, we infer that these four genes form the critical core of the functional module or were the first to assemble. This inference is supported by the occurrence of the same four-gene cluster within two taxa of the order *Lactobacillales*, *Lactobacillus plantarum* and *Enterococcus faecalis* (55). This restricted occurrence in a related but distinct lineage is consistent with the horizontal transfer of the *secG-yvaK-rnr-smpB* cluster from the order *Bacillales* (42). If we are correct in our inference that the cluster forms a functional module with an important role in cold adaptation, its presence in pathogens such as *B. cereus* and *L. monocytogenes* likely contributes to their ability to grow at refrigerator temperatures and cause significant food-borne illness.

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